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Fumonisin and beauvericin induce apoptosis in turkey peripheral blood lymphocytes

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Abstract

Fumonisins, a family of mycotoxins produced by *Fusarium verticillioides* (synonym *Fusarium moniliforme* Sheldon) and *F. proliferatum*, have been associated with various deleterious effects in different animal species. Serological, hematological and pathological effects and mortality have previously been observed in broiler chicks fed *F. proliferatum* culture material containing known concentrations of fumonisin, moniliformin and beauvericin. Turkey peripheral blood lymphocytes were exposed *in vitro* for 72 hours to fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), hydrolyzed fumonisin B_1 (HFB_1), moniliformin and tricarballylic acid (TCA) (0.01-25 μg/ml). A decrease in cell proliferation, as determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] bioassay, occurred in the order: $FB_2 > FB_1 > HFB_1$, with $IC_{50} = 0.6$ μM, 1 μM and 10 μM, respectively. Internucleosomal DNA fragmentation and morphological features characteristic of apoptosis were observed following exposure to fumonisin B_1 and beauvericin; cytoplasmic condensation and membrane blebbing were seen by light microscopy. Tricarballylic acid and moniliformin did not interfere with cell proliferation. Results suggested that fumonisin B_1 and beauvericin may affect immune functions by suppressing proliferation and inducing apoptosis of lymphocytes.

Key words: apoptosis, beauvericin, fumonisin, *Fusarium proliferatum*, inhibition of proliferation, moniliformin

Introduction

Fusarium verticillioides (synonym *F. moniliforme* Sheldon) and a related species, *F. proliferatum*, frequently found as contaminants in feed containing corn or corn screenings, are capable of producing high levels of the fumonisin mycotoxins. Fumonisins have been associated with diseases in animals: equine leukoencephalomalacia (ELEM), porcine pulmonary edema and liver cancer in rats [1, 2]. Reports of the association of ELEM with mold-damaged corn have existed for 100 years, although the structural elucidation of fumonisins was only described in 1988 [3]. Fumonisin B_1 (FB_1) can cause inhibition of sphingomyelin biosynthesis in rat hepatocyte culture by inhibiting sphinganine *N*-acyltransferase (ceramide synthase) in the *de novo* sphingolipid pathway, resulting in an accumulation of free sphinganine [4]. In animals, exposure to fumonisin causes an increase in free sphinganine, an increase in the ratio of free sphinganine:sphingosine and a decrease in complex sphingolipids [5]. Both purified toxins and *F. verticillioides* or *F. proliferatum* culture material can produce this effect.

Four fumonisin B analogs (FB_{1-4}) have been identified [6]; FB_1 is the major form occurring both in nature and in culture. Although studies have utilized *F. proliferatum* culture material to test the effects of fumonisins, it is difficult to ascribe effects solely to fumonisin(s) because other mycotoxins (moniliformin and beauvericin) are frequently produced [7, 8]. Another fumonisin compound of interest is hydrolyzed fumonisin B_1 (HFB_1), which is formed during the production of masa and tortillas when the tricarballylic sidechains of FB_1 are cleaved by alkaline hydrolysis (nixtamalization) [9].

Recent research in several laboratories has demonstrated fumonisin-induced apoptosis in different systems. In CV-1 (monkey kidney) cells, FB_1 induced

the stereotypical hallmarks of apoptosis, including the formation of DNA ladders, compaction of nuclear DNA and the subsequent appearance of apoptotic bodies [10]. Neonatal human keratinocytes and human esophageal epithelial cells have developed morphological features consistent with apoptosis when treated with FB_1 [11]. In a porcine kidney epithelial cell line, FB_1 produced morphological changes of cell shrinking and membrane blebbing, indicating that the mycotoxin induced apoptosis [12].

In prior research, the cytotoxic effects of FB_1 and FB_2 in turkey lymphocytes were examined [13]. In the present study, turkey peripheral blood mononuclear cells (PBMC) were exposed to FB_1, FB_2, HFB_1, tricarballylic acid (TCA), moniliformin or beauvericin to determine if a dose-dependent inhibition of proliferation occurred and if apoptotic cell death was induced. PBMC were used in this study because they represent native cells rather than an established cell line. The cells were primarily a mixture of lymphocytes, with some monocytes that had not been completely removed by adherence. Morphological changes indicative of apoptosis were noted by microscopic examination of cells. The most striking observation was the projection of knobby protuberances at the lymphocyte cell surface, giving the impression that the cells were boiling. The term zeosis, rapid blebbing, is characteristic of the type of bubbling in which relatively rapid projection and retraction of the cytoplasm occurs [14]. The apoptosis induced in lymphocytes following *in vitro* exposure may indicate that similar effects can occur in animals following exposure to fumonisin and beauvericin.

Materials and methods

Mycotoxins and related microbial products

Fumonisin B_1, FB_2, moniliformin, beauvericin and TCA were purchased from Sigma Chemical Company (St. Louis, MO). The fumonisins and beauvericin were also produced by *F. proliferatum* M-5991 and isolated [8]. Hydrolyzed fumonisin B_1 was prepared by base hydrolysis of FB_1 and was a gift from G. A. Bennett (NCAUR).

For production of HFB_1, fumonisins were extracted from culture material (*F. proliferatum* grown on rice) (50 g) with acetonitrile-water (50:50) (250 ml) and filtered. The filtrate (100 ml) was subjected to alkaline hydrolysis (50 ml 2N NaOH) at 60-65 °C for 1 hr. After neutralization with 2N HCl, the extract was added to an Amberlite XAD-2 column (Sigma). HFB_1 was eluted from the column with acetonitrile-water (60:40) and relative purity was determined by TLC. The aminopentol product was further purified using a C_{18} SPE column (Mega Bond Elut, Varian Sample Preparation Products, Harbor City, CA). Purity of HFB1(>98%) was determined, following derivatization, by HPLC (fluorescence detection) relative to a known standard [8].

Cell culture and cytotoxicity test

Turkey peripheral blood lymphocytes were obtained from heparinized whole blood collected from the wing vein of 12 and 14 weeks old Nicholas (broad-breasted white) turkeys. Blood was diluted with an equal volume of Hank's balanced salt solution (HBSS). Four ml of diluted blood were layered onto a Percoll gradient (2 ml 45% plus 2 ml 54% Percoll) and centrifuged at 500 g for 20 min. The band of lymphocytes was removed and cells were washed 2X with HBSS. Following the last centrifugation, cells were resuspended in RPMI 1640 (25 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acids, 100 units penicillin/ml, 0.1 mg streptomycin/ml, 0.25 μg amphotericin B/ml) containing 5% heat-inactivated turkey serum (TS). Cells were diluted using a NOVA Celltrack 6 diluter, counted with a NOVA Celltrack2 analyzer (Nova Biomedical Corp., Waltham, MA) and adjusted to a concentration of 2×10^6 lymphocytes/ml. Lymphocytes were exposed to FB_1, FB_2, HFB_1, moniliformin, or tricarballylic acid (1,2,3-Propanetricarboxylic acid) (0.01–25 μg/ml) for 72 hr at 39 °C, 5% CO_2, in 96-well microtiter plates for the MTT bioassay [13, 15].

Two-fold dilutions of FB_1, FB_2, HFB_1, moniliformin and TCA (0.01-25 μg/ml) were made in RPMI-TS in 96-well, flat bottom microtiter plates. To 100 μl diluted toxin was added 100 μl cell suspension. Controls without toxin and MTT were included. After incubation for 72 hr, twenty μl MTT (5 mg/ml) were added and plates were incubated for four additional hours. Plates were centrifuged at 1600 g for 20 min and supernatant was removed. One hundred μl dimethylsulfoxide (DMSO) were added to the pelleted cells. The plates were shaken for ten minutes and absorbance at 540 nm measured on a microplate reader (Dynatech MR700, Dynatech Laboratories, Inc., Chantilly, VA). Mean values of quadrupli-

cates at each toxin concentration were compared with corresponding controls.

Cells were also incubated in the presence of FB_1, FB_2, HFB_1, moniliformin or beauvericin (8 μM and 50 μM) for 72 hr in 25 cm^2 vented culture flasks and photographed either unstained or following staining with Diff-Quick (Wright's Giemsa) stain set (Baxter Healthcare Corp., McGaw Park, IL).

DNA fragmentation

Cells were incubated in the presence of FB_1, FB_2, HFB_1, moniliformin or beauvericin (8 μM and 50 μM) for 72 hr in 25 cm^2 vented culture flasks for DNA isolation. A ten ml volume of mycotoxin-exposed lymphocytes was centrifuged at 400 g for 10 min. The supernatant was discarded and the cells were lyophilized. Components of the Magic DNA Purification Systems (Promega, Madison, WI) were used for DNA isolation. The lyophilized cells (approx. 10^4) were resuspended in 200 μl Cell Resuspension Solution (50 mM Tris, pH 7.5, 10 mM EDTA, 100 μg/ml RNase A) and lysed by adding 200 μl 1% SDS; 200 μl Neutralization Solution (2.55 M potassium acetate) (pH 4.8) were added and the tubes were centrifuged at 10,000 g for 10 min. The supernatants were transferred to microfuge tubes and Gene Clean II (Bio 101, Inc., La Jolla, CA) was used for DNA purification. DNA fragments were separated by agarose gel electrophoresis using 2% Nusieve (FMC)/1% Ultra Pure Agarose (BRL) in 1X TPE (90 mM Tris-phosphate, 2 mM EDTA). One hundred base pair DNA molecular weight markers (Cat. No. 156628-019, GIBCO BRL, Grand Island, NY) were used for determining the size of the fragmented chromosomal DNA. The gels (75 × 50 mm) were run for 45 min at 90 V, stained for 30 min at room temperature using ethidium bromide, and photographed under ultraviolet illumination.

Results

Turkey peripheral blood lymphocytes which had been exposed *in vitro* to FB_1, FB_2 or HFB_1 (0.01-25 μg/ml) for 72 hr exhibited a dose-dependent decrease in ability to proliferate. FB_1 (IC_{50} = 1 μM) and HFB_1 (IC_{50} = 10 μM)(Figure 1) and FB_2 (IC_{50} = 0.6 μM) [13] caused inhibition of proliferation. The IC_{50} value represents 50% inhibition. This did not represent only increased cell death with toxin-exposed cells because control cells have been shown to have a linear increase

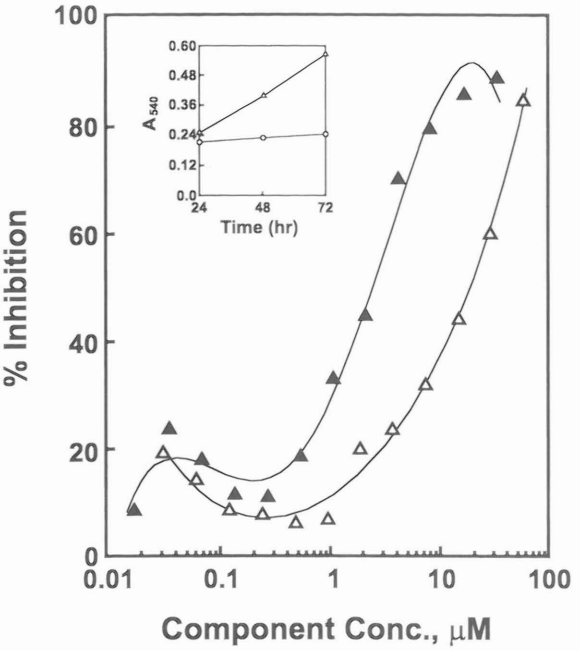

Figure 1. Effect of FB_1 and HFB_1 on proliferation of turkey peripheral blood lymphocytes, as determined by the MTT bioassay. Cells were incubated with FB_1 (▲) or HFB_1 (△) (0.01-25 μg/ml) for 72 hr at 39 °C. Each point is the mean of 4 replicates. Percent inhibition was calculated relative to control cells [12]. Shown in the insert is the difference in proliferation observed between control cells (△) and cells exposed to FB_2 (○), monitored as absorbance at 540 nm at 24, 48 and 72 hr.

in absorbance over 24–72 h [13]. A comparison of control cells and cells exposed to FB_2 is shown as an insert in Figure 1. A similar level of inhibition of proliferation of turkey lymphocytes had been observed when cells were exposed to FB_1, stimulated with 5 μg concanavalin A/ml and uptake of ^{3}H-thymidine was measured (unpublished data). Cells exposed to moniliformin or TCA resembled control cells and did not show inhibition of proliferation.

The present evidence indicated that turkey peripheral mononuclear cells underwent apoptosis when treated *in vitro* with FB_1, HFB_1, FB_2 and beauvericin. Evidence of apoptosis was observed in several different manners, including characteristic DNA fragmentation (cleavage of DNA between histone octamers to generate a ladder of fragments) and light microscopy. Nuclear DNA fragmentation was assessed qualitatively by agarose gel electrophoresis. DNA fragmentation (multiples of approx. 180 base pairs) was observed in cells exposed to 8 μM FB_1 (Figure 2A and 2B), 50 μM and 8 μM beauvericin (Figure 2A and 2B), and 8 μM FB1 plus 8 μM beauvericin

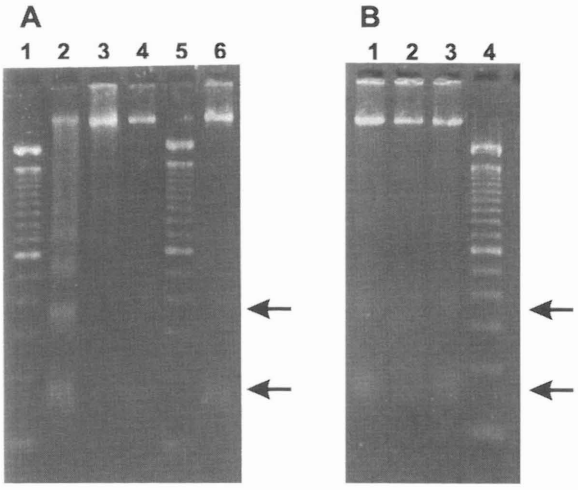

Figure 2. Electrophoretic gel laddering of cellular DNA isolated from turkey peripheral blood lymphocytes cultured for 72 hr with FB_1, beauvericin or FB_1 and beauvericin combined. DNA was isolated, loaded onto the agarose gel and analyzed. **A:** Represented in lanes 1 through 6 are the following:1 and 5, molecular weight markers (100-base pair ladder of DNA); 2, cells treated with beauvericin (50 μM); 3, untreated cells (control); 4 and 6, cells treated with FB_1 (8 μM). **B:** Cells represented in lanes 1 through 3 were treated as follows: 1, FB_1 (8 μM); 2, beauvericin (8 μM); 3, FB_1 (8 μM) and beauvericin (8 μM). Molecular weight markers are in lane 4.

(Figure 2B), as well as with FB_2 or HFB_1 (data not shown) at 8 μM and 50 μM. No DNA fragmentation occurred with exposure to moniliformin. The effect of exposure to FB_1 and beauvericin (Figure 2B) together was slightly additive. Exposure to FB_1 (Figure 2B) showed more DNA fragmentation occurred than with beauvericin (Figure 2B). Indications of apoptosis were visible in unstained cells that had been exposed to FB_1, FB_2, HFB_1 and beauvericin (Figures 3 and 4) at 8 μM and 50 μM for 72 hr. Morphological changes characteristic of apoptosis seen by light microscopy included condensation of chromatin, with highly condensed chromatin forming crescents around the periphery of the cells and displaced nuclei and blebs (high levels of cytoplasmic vacuolization). The number of cells remaining after exposure to 50 μM of FB_1, FB_2, HFB_1 or beauvericin was reduced, compared to those exposed to 8 μM of these toxins (Figures 3 and 4). There were fewer cells in FB_2-exposed cultures (Figure 3B) than in FB_1- and HFB_1-exposed cultures (Figure 3A and C, respectively) at 50 μM levels. There were more cells present following exposure to beauvericin (50 μM) (Figure 4D), than with FB_1, FB_2 or HFB_1 (50 μM) (Figure 3A, B and C).

Vacuoles and condensation of nuclear and cytoplasmic components of cells exposed to 8 μM of FB_1, FB_2 or HFB_1 were clearly visible when cells stained with Wright's Giemsa stain were examined by light microscopy (Figure 5A, B and C, respectively), compared to control cells. Exposure to moniliformin or TCA did not appear to induce apoptosis under the conditions used.

Discussion

This is the first study to report the occurrence of zeosis (rapid blebbing) after separate exposure to fumonisins and beauvericin. Eukaryotic cells die either by necrosis, in which DNA remains intact, or by apoptosis [16], in which DNA is cleaved into nucleosome-length fragments; the formation of DNA fragments of oligonucleosomal lengths (180–200 bp) is a biochemical characteristic of apoptosis in many types of cells [17]. In the later stages of apoptosis, internucleosomal cleavage of DNA generates both monomers and multimers of DNA. Apoptotic lymphocytes also lose membrane phospholipid asymmetry, leading to the exposure of phosphatidylserine on the outer leaflet of the plasma membrane, triggering specific recognition and phagocytosis by macrophages [18].

The lymphocytes used in these studies were obtained from turkeys at a local commercial farm where 5,000 birds live in a large enclosed area. No mitogens were used in the *in vitro* tests. High levels of mitogenic or antigenic material (perhaps superantigens) may have been present in the turkey blood. Superantigens have the ability to interact with a large proportion of the T cell repertoire. Instead of the 1 in 10^4 to 1 in 10^6 T cells reacting in an MHC-restricted fashion, up to 30% of T cells can interact with a superantigen [19]. This may explain the high degree of lymphocyte proliferation observed with the control cells, even though mitogens were not added exogenously [13].

Turkey peripheral blood lymphocytes were prevented from proliferating by *in vitro* exposure to FB_1, HFB_1 or FB_2 (Figure 1). The degree of inhibition observed with HFB_1 and FB_1, with FB_1 being a more potent inhibitor, was similar to the five- to ten-fold difference reported for hepatocytes and a human colonic cell line exposed to HFB_1 and FB_1 when assayed for their ability to inhibit ceramide synthase [20, 21].

Lymphocytes exposed to FB_1 or beauvericin (Figure 2A and B) or FB_1 and beauvericin (Figure 2B) all exhibited DNA fragmentation and nuclear condensation characteristic of apoptosis. Certain instances of toxin exposure may share the morphology and bio-

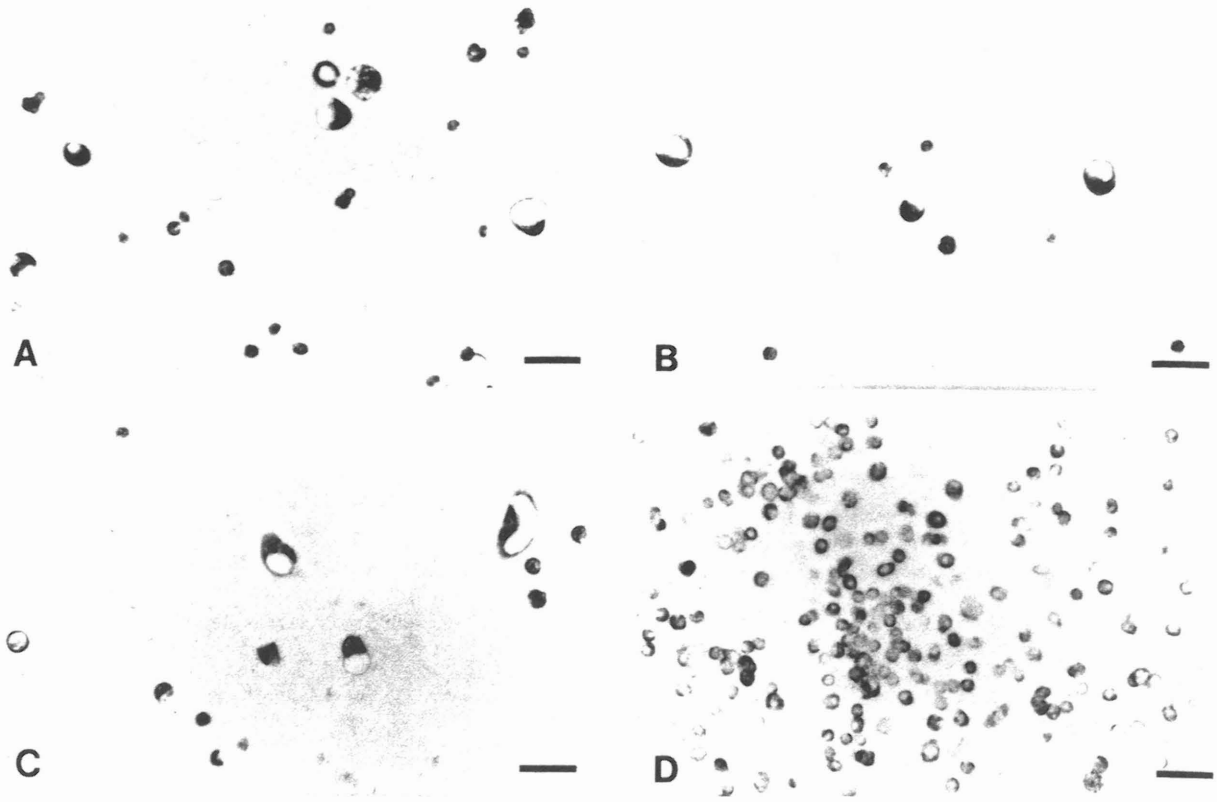

Figure 3. Turkey lymphocytes following incubation in the presence of toxins for 72 hr at 39 °C. Unstained cells were photographed using an inverted microscope. **A**: 50 μM FB_1; **B**: 50 μM FB_2; **C**: 50 μM HFB_1; **D**: Control. Fewer cells were present in FB_2-exposed cultures (**B**) than in FB_1- and HFB_1- exposed cultures (**A** and **C**, respectively). The calibration bars are 25 μm.

chemical changes characteristic of apoptosis [14]. Apparently, only certain populations of cells were undergoing apoptosis; resting lymphocytes were observed near apoptotic cells (Figures 3, 4 and 5). Sensitivity to fumonisins appeared to be restricted to proliferating cells, which may be more susceptible to chemical toxicity because they have a more active metabolism [22]. Similar results were observed with the antiproliferative activity of myriocin, which was specific for antigen-stimulated lymphocytes [23]. Inhibition of proliferation is not readily observed at 24 h of incubation [13]. In this study, further incubation was required to detect apoptotic cells.

Beauvericin, an ionophore structurally related to valinomycin, can trigger an increase in cytoplasmic calcium concentration and induce apoptosis in cultured cells [24]. Certain strains of *F. proliferatum* and *F. subglutinans* produce beauvericin, a cyclic hexadepsipeptide composed of alternating *N*-methyl-*L*-phenylalanine and D-α-hydroxyisovaleric acid residues. Beauvericin is also produced by *Beauveria bassiana*, a promising mycoinsecticide for biocontrol of domestic and agricultural pests [25]. A variety of studies examining fumonisin toxicity have utilized *F. proliferatum* culture material, without realizing that high levels of beauvericin may also have been present [8].

Moniliformin, a water-soluble sodium or potassium salt of cyclobutenedione, was initially described as being produced by *F. moniliforme* [26]. Subsequent reports have indicated that *F. proliferatum*, but not *F. moniliforme*, is capable of producing moniliformin, which has been associated with acute toxicity in experimental animals. Moniliformin appears to act as a suicide enzyme inactivator, interacting with thiamine pyrophosphate to cause inhibition of pyruvate dehydrogenase [27]. Thiamine deficiencies in young chicks fed moldy feed may have been due to the presence of moniliformin [28]. It is not surprising that toxic effects due to moniliformin were not observed in the present study. Culture media can contain levels of thiamine that would be protective. For example, the thiamine concentration of normal culture

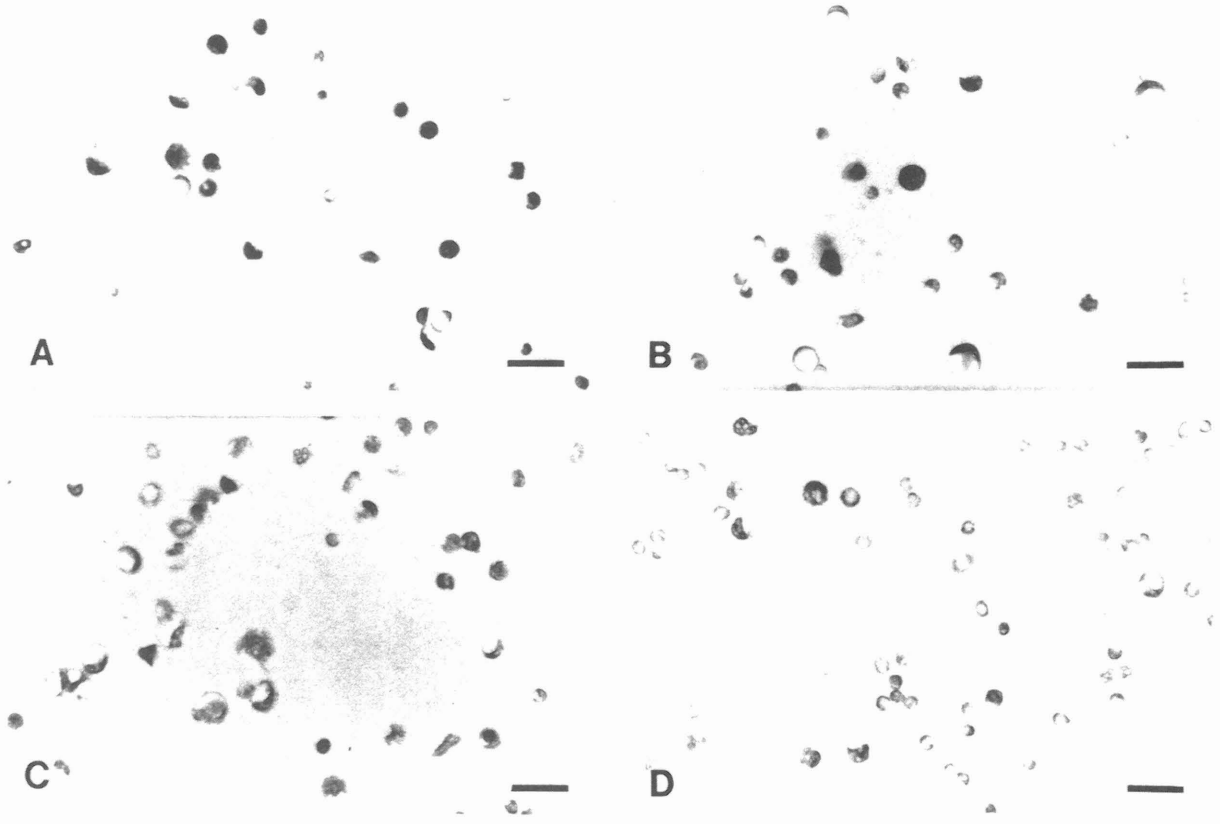

Figure 4. Turkey lymphocytes following incubation in the presence of toxins for 72 hr at 39 °C. Unstained cells were photographed using an inverted microscope. **A**: 8 μM FB_1; **B**: 8 μM FB_2; **C**: 8 μM HFB_1; **D**: 50 μM beauvericin. The calibration bars are 25 μm.

medium is 10 μM, but physiological levels of thiamine (<1 μM) are much lower [29].

The appearance of the lymphocytes after *in vitro* exposure to FB_1, FB_2, HFB_1 and beauvericin indicated that the cells were undergoing apoptosis. In the presence of these toxins, cells exhibited vacuolization (Figures 3, 4 and 5). The most consistent hematological change noted in rats receiving an intraperitoneal injection of FB_1 was an increase in vacuolated bone marrow cells [30]. Nitric oxide may play a role in the observed apoptosis of lymphocytes. Recent research involving exposure of rat splenic cells to FB_1 indicates that nitric oxide produced by macrophages may be responsible for the apoptotic death of lymphocytes [31]. A small number of macrophages were present in the lymphocyte populations in this study. Results indicate that fumonisins and beauvericin may be able to cause immunosuppression in animals by abrogating proliferation and inducing apoptosis.

In conclusion, the major points made in this study were that FB_1, FB_2, HFB_1, and beauvericin were capable of suppressing proliferation of lymphocytes and inducing apoptosis. When FB_1 and beauvericin were present together at 8 μM, a slight additive effect was observed. Moniliformin and TCA did not induce apoptosis under the conditions used in the study. It is hypothesized that apoptosis may be triggered by the production of nitric oxide, as has been described with rat splenic cells exposed to FB_1 [31].

Acknowledgment

I wish to acknowledge the generous gift of purified fumonisins from Ronald D. Plattner. *Fusarium proliferatum* M-5991 was obtained from the Fusarium Research Center, The Pennsylvania State University.

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the products, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

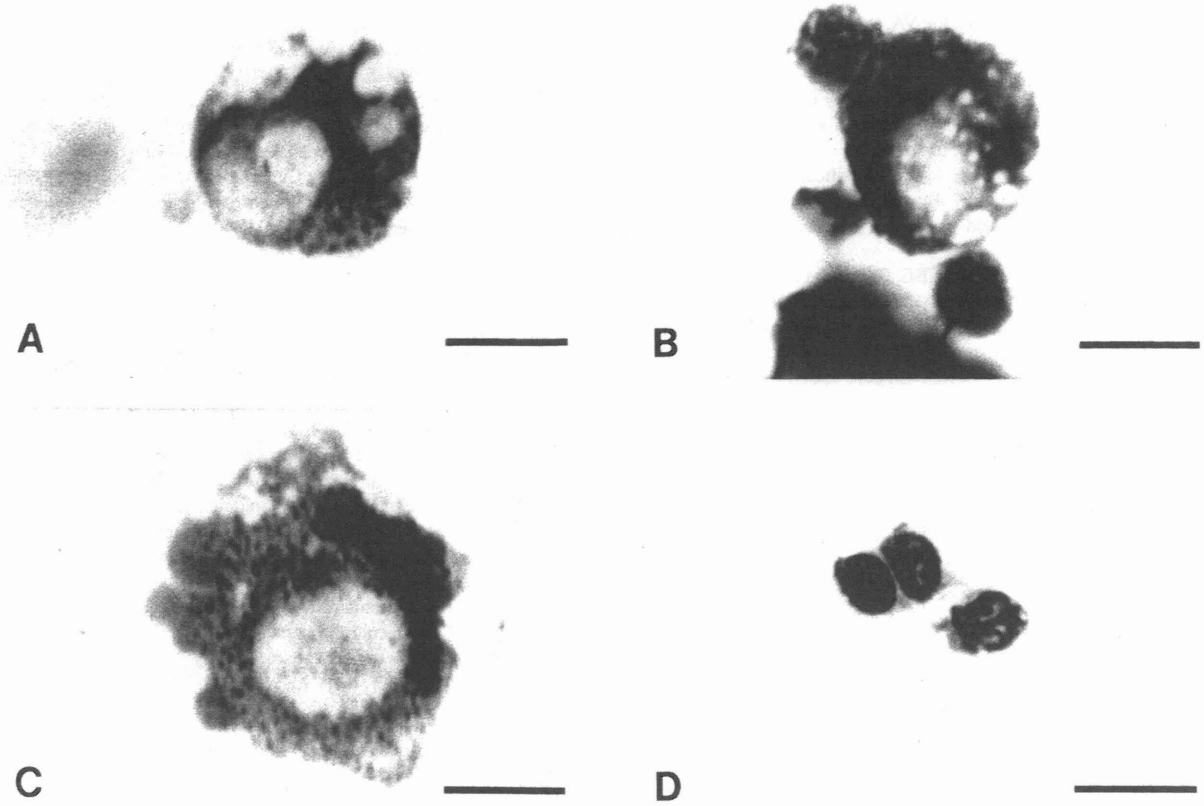

Figure 5. Turkey lymphocytes following incubation in the presence of toxins for 72 hr at 39 °C. Cells were stained with Wright's Giemsa stain and photographed. **A**: 8 μM FB_1; **B**: 8 μM FB_2; **C**: 8 μM HFB_1; **D**: Control. Prominent vacuoles were observed in fumonisin-treated cells (**A**–**C**). Quiescent cells were observed near an apoptotic cell (**B**). The calibration bars are 10 μm.

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